APPENDIX B

Chapter 108
Schemata for the production of monoclonal antibody-producing hybridomas

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The capacity to generate antigen-specific monoclonal antibody reagents has attracted the attention of immunologists for well over three decades. The advantage of having access to large quantities of monospecific immunoglobulin for tools in research, serology or immunotherapy can hardly be overstated. Early attempts, however, to discover specific antigens for any one of the many immunoglobulins produced by spontaneously arising myelomas were generally unsuccessful [1]. In addition, numerous trials with test animals to induce myeloma protein that had specific antigen binding activity were equally frustrating [2].

The introduction of monoclonal antibody technology brought about a revolution in immune serology. The capacity to fuse immunized B lymphocytes with cells from a cultured line and subsequently to select hybridomas that secrete immunoglobulin of desired specificity has made available unlimited quantities of highly useful monoclonal antibody reagents. Since the introduction of hybridomas less than a decade ago, numerous articles have been published concerning the production or use of monoclonal reagents. This chapter reviews only a limited number of these articles, discussing the basics of hybridoma formation and highlighting several useful techniques for selecting desired hybridomas of mouse or human origin.

The production of murine hybridomas

The concept of constructing hybridomas to immortalize B cells secreting specific antibody developed from research into the synthesis and production of antibody by fused myeloma cell lines. Using inactivated Sendai virus to fuse two antibody-producing cell lines of rat and mouse origin, Cotton & Milstein created stable hybrid cells which expressed the immunoglobulin products of both parental myelomas [3]. After reproducing these fusion experiments between two myeloma cell lines of mouse origin, Milstein & Kohler successfully fused myeloma cells with spleen cells obtained from a mouse immunized with sheep red blood cells (SRBC) [4]. From this fusion these investigators selected clones of cells with the desired phenotypic traits of both cell types, namely the ability to grow in culture from the parent myeloma line and the capacity for specific anti-SRBC antibody secretion from the immune-responsive B cell. These hybridomas retained their specific-antibody secretion activity even after many months in culture.

The success of these experiments relied upon the elimination of unfused cells. Although spleen cells are unable to grow in culture, unfused myeloma cells will soon overgrow the small percentage of newly generated hybridomas unless they can be selectively excluded. For this, the myeloma cell line is first made genetically defective in its salvage nucleotide biosynthetic pathways by selecting for mutant cells that are resistant to either 8-azaguanine, 6-thioguanine, or 5-bromo-2′-deoxyuridine. A cell line with such a defect is unable to grow in Littlefield's 'HAT' medium, containing hypoxanthine, aminopterin and thymidine [5]. As the aminopterin blocks *de novo* biosynthesis of purines and pyrimidines, a cell's survival in HAT is dependent upon its ability to utilize the exogenous hypoxanthine and thymidine through these salvage biosynthetic pathways. Thus, for the experiment described above, Milstein & Kohler used a myeloma cell line selected for resistance to 8-azaguanine. This line was consequently found to have a deficiency in the enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRTase), making it unable to grow in HAT medium. Fusion of these cells with normal spleen cells which possess this enzyme, however, produces hybridomas resistant to HAT selection.

Myeloma lines suitable for fusion

Improvements in the myeloma cell line used for production of hybridomas has facilitated the creation of antigen-specific, monoclonal-producing cell lines. The first experiments were performed with P3-X-63-Ag8, a clonal line of the BALB/c myeloma MOPC-21, as described above [3]. Although amenable to HAT selection, this cell line would often form hybrids with spleen cells that secreted a variety of tetrameric molecules composed of mixtures of the heavy and light chains of both the immortalized splenocyte and the immunoglobulin chains of the IgG1 κ MOPC-21 myeloma protein. It was, therefore, often necessary to select variant hybrids that had lost the ability to synthesize the heavy or heavy and light chains of the MOPC-21 protein; because of this, a myeloma cell line P3-NS1-1, abbreviated NS-1, was developed from the original P3-X-63-Ag8. This line had lost the ability to produce the IgG1 heavy chain [6]. Subsequently, two mouse myeloma lines, Sp2/0-Ag14 and P3X63-Ag8.653, were developed that are total non-producers of immunoglobulin [7,8]. As they do not synthesize either light or heavy chains, the hybrid cells derived with these parental cell lines only produce antibody of the spleen cell parent.

Whatever line is chosen for cell fusion partner, it is important to have the cells growing exponentially prior to cell fusion. The myeloma lines mentioned above grow to a maximum density of $3–6 \times 10^5$ cells/ml with a doubling time of 16–26 h. After this density is reached, there is a rapid fall in cell viability as these cells enter stationary phase. Myeloma cells with viability of less than 90% are unacceptable fusion partners.

Immunization protocol

Immunization protocols for generating immune lymphocytes for hybrid formation are varied, but share a few important principles. The optimal route of immunization, amount of antigen used and the timing between injections must be determined for each antigen. Generally, hyperimmunization is not necessary [9]. A typical procedure for generating immune splenocytes to cellular antigens is to prime mice with approximately 2×10^7 cells injected into the peritoneum. The mice receive booster injections with the same number of cells administered i.p. 14–21 days later. For sensitization to soluble antigen, mice are immunized with antigen precipitated in alum and mixed with 2×10^9 killed *Bordetella pertussis* organisms. Animals are subsequently boosted with aqueous antigen without *B. pertussis* 1–3 weeks later. A critical feature of all immunization protocols, however, is the timing of the final antigen boost relative to the time of cell fusion. This boost should be delivered 3 days prior to removing the spleen for cell hybridization with the myeloma line. In a series of early hybridization experiments, Kohler found that the yield of specific, antibody-producing hybridomas increased up to 3 days after the booster injection and subsequently decreased.

In lieu of whole cells, membrane extract may sometimes be used to immunize donor mice. Membrane proteins are extracted with Nonidet P40 (NP40) detergent in 10 mM-Tris-Cl (pH 8), 0.15 M-NaCl, and 0.02% NaN_3 (extraction buffer). For this, 0.5 ml of cold extraction buffer is added to a pellet of 10^7 washed cells that is vortexed vigorously. After a 15 min incubation at 4 °C, the suspension is spun at 27 000 *g* for 20 min to pellet nuclei from the disrupted cells. The harvested supernate is dialysed extensively against phosphate-buffered saline at pH 7.4 prior to injection into mice. Generally a 0.5% concentration of NP40 works best for the extraction of a number of lymphocyte antigens, including immunoglobulin, differentiation antigens and antigens coded for by the major histocompatibility gene complex. However, extraction of other antigens may require different concentrations of NP40.

An alternative to NP40 extraction is the method of hypotonic cytolysis in sucrose [10,11]. For this, 10^9 cells are suspended in 10 ml of 0.32 M-sucrose (pH 7.4) and disrupted in a cell homogenizer. Nuclei and cell debris are pelleted from the cell homogenate by two sequential 20 min centrifugations of the supernatant at 400 *g* and then 4000 *g* at 4 °C. The membrane pellet is recovered by spinning the supernatant for 1 h at 20 000 *g*. This pellet is resuspended in 10 ml of phosphate-buffered saline and stored at −70 °C until required for immunization.

For many antigens, special measures must be taken to increase the yield of hybridomas secreting monoclonal antibody of the desired specificity and/or affinity. Stahli *et al.*, for example, found it necessary to hyperimmunize mice with the protein hormone human choriogonadotrophin (hCG) by repeated injections with the protein in complete Freund's adjuvant (CFA) [12]. Mice were screened for specific antibody production by solid-phase radioimmunoassay and animals with high titres of specific circulating antibody were selected for fusion. Final immunization was without adjuvant and required large amounts of antigen injected i.v. in 200 μg aliquots on each of three successive days, beginning on day −4 prior to fusion. Similar immunization regimens have succeeded in producing hybridomas to the human leucocyte interferons [13]. To monitor for effective stimulation of B-cell clones, these investigators analysed spleen cell

populations prior to fusion for forward-angle light scatter using a flow cytometer. Noting that lymphocytes with higher values of light scatter represent B-cell blasts stimulated by the final immunization, they demonstrated a linear correlation between the yield of specific hybridomas and the percentage of large cells noted in the spleen cell population prior to fusion with the myeloma line [14].

Adoptive transfer techniques may also increase the yield of desired hybridomas. Fox *et al.* noted a 10- to 50-fold increase in the percentage of antigen-specific, antibody-producing hybridomas by adoptively transferring spleen cells from hyperimmunized donors into lethally irradiated syngeneic mice [15]. For this, donor mice were first immunized with antigen in CFA at multiple sites s.c. Mice were boosted at 3–4 week intervals with antigen in incomplete Freund's adjuvant (IFA) delivered i.p. Ten days after the third or fourth injection, mice with the highest titres of specific antibody are sacrificed and their spleens removed. Between 30 and 50×10^6 spleen cells are adoptively transferred into syngeneic recipients lethally X-irradiated with 550 rads 24 h prior to cell transfer. Immediately after transfer, the mice receive the immunizing antigen i.p. in 0.5 ml of 0.15 M-saline without adjuvant. Four days later, their spleens are removed for fusion with the appropriate myeloma cell line.

The purity of the immunogen is an important factor in determining the final yield of antigen-specific hybridomas. Immunization with crude preparations will result in the stimulation of many B-cell clones producing antibody to specificities other than those on the antigen of interest. Thus, efforts to purify the antigen prior to immunization will help to yield a higher percentage of hybridomas producing antibody of desired specificity. Recently, a number of investigators have made use of this principle to obtain new monoclonal antibodies specific for certain cell surface antigens. Membrane extracts from cells are prepared as outlined above. Prior to immunization, however, the extract is passed over a solid-phase immunoabsorbant of existing monoclonal antibodies with specificity for cell surface antigens. Through this procedure, surface molecules not recognized by existing monoclonal antibodies may be extracted from the more immunogenic surface antigens. Hyperimmunization with such extracts and subsequent hybridization enhances the chance of obtaining hybridomas which produce monoclonal antibody with novel antigenic specificity.

Fusion protocol

The fusing agent most commonly used is polyethylene glycol (PEG) [16]. Although Milstein *et al.* utilized inactivated Sendai virus to form the first hybridomas, PEG was soon recognized as being superior to virus in both availability and consistency. Both PEG with molecular weights 1000 (PEG 1000) and 1500 (PEG 1500) are adequate for cell fusion. Both types should be odourless and white. For fusion, PEG is autoclaved for 15 min and kept at 56 °C. The optimal concentration of PEG will depend on the cell lines and lot of PEG, solutions generally ranging from 35% (v/v) to 50%. This is prepared by adding an appropriate volume of heated PEG to serum-free culture medium that is then kept at 37 °C prior to hybridization. The optimal concentration of PEG needs to be determined empirically.

Procedure

1 A mixture of 10^8 immunized spleen cells and 10^8 myeloma cells in serum-free RPMI-1640 is pelleted in a 50 ml conical-bottomed polypropylene-plastic centrifuge tube at 400 *g* for 10 min. The medium is removed as completely as possible and the fusion initiated at 37 °C by the gradual addition over a 1 min period of 1 ml of PEG solution in serum-free medium. During this time the cells are suspended by gentle stirring with the tip of the pipette used to deliver the heated PEG solution.

2 After an additional minute of stirring at 37 °C, 1 ml of pre-warmed serum free medium is gradually added to the stirring suspension over the course of 1 min.

3 Step 2 is repeated with another millilitre of serum-free medium.

4 Finally, 7 ml of serum-free medium is added to the suspension over a 2–3 min period with continuous stirring.

5 The mixture is subsequently pelleted at 400 *g* for 10 min at room temperature and the supernatant removed.

6 Ten millilitres of medium with 10% FCS is directly released on to the pellet, which is suspended with minimal use of a wide-mouthed pipette so as not to disrupt newly formed hybrids. An additional 10 ml of warm medium with FCS is added to the tube and the contents are swirled.

7 The cells are subsequently cultured at an initial concentration of $5–10 \times 10^6$/ml in non-selective medium at 37 °C in 7% CO_2 [9].

8 On the day after the fusion, the cell cultures are fed with an equal volume of culture medium, containing 100 μM-hypoxanthine, 10 μM-aminopterin, and 30 μM-thymidine (HAT medium). This medium is made by adding the appropriate volume of a filter-sterilized 50× HAT solution previously prepared in triple-distilled H_2O and stored at −20 °C.

9 On days 2, 3, 5, 8, and 11 one-half of the medium

from each culture well is aspirated, and an equal volume of fresh HAT medium is added.

10 After day 11, the culture fluid is exchanged every 3–4 days until hybrid colonies are identified, at which time the cultures are fed HAT medium lacking aminopterin (HT).

11 After 1 week's culture in HT medium, when the amount of residual aminopterin in the cell culture medium is negligible, the cells can be propagated in tissue culture medium lacking exogenous hypoxanthine and thymidine.

Production of hybridomas producing human monoclonal antibody

Cell lines for hybridoma production

The first human monoclonal-producing hybridomas were derived from fusions between human lymphoid cells and the cultured murine myeloma cell line, NS-1. Using fusion protocols similar to that described above, several groups succeeded in producing human–mouse hybrids that secreted human monoclonal antibody [17–22]. In contrast to murine and mouse–rat hybridomas [23], however, human–mouse hybrids are very unstable, these cells rapidly losing human chromosomes in successive generations after cell fusion [24]. As the genes coding for light and heavy chains are on separate chromosomes, at least two human chromosomes must be retained in the progeny of such hybrid cells to allow for the continued expression of the intact human immunoglobulin molecule. Although chromosome 14, on which are located the genes for the human antibody heavy chain [25], is preferentially retained in successive generation of mouse–human hybrids, chromosomes 2 and 22, on which are found the genes coding for the κ and λ light chains respectively [26,27], are lost rapidly in successive generations, resulting in unstable production of whole human immunoglobulin.

In order to overcome the instability of human–mouse hybridomas, a number of investigators have each developed cultured human lines for the generation of human–human hybridomas [28–36]. A representative listing of such lines is presented in Table 108.1. These lines have all been developed for sensitivity to HAT medium either by selecting for resistance to 8-azaguanine (8-AZ) or 6-thioguanine (6-TG). Most of the cell lines are Epstein–Barr virus (EBV) transformed lymphoblastoid cell lines, owing to the extreme difficulty in adapting human plasmacytomas to *in vitro* culture. Several investigators have published comparative studies, addressing the efficiency with which the various human cell lines fuse with B-lineage lymphocytes and form stable antibody-secreting hybridomas [37–39]. Despite the advent of human cell lines suitable for cell fusion, however, antigen-specific, monoclonal antibody-producing human–human hybridomas generally remain more difficult to produce and less stable than rat or mouse hybridomas. Another limitation is the amount of monoclonal antibody secreted by the human hybridomas. This amount is generally in the order of 10–100 ng antibody per millilitre of culture supernatant compared to over 10 μg/ml in supernatants from rat or mouse hybridomas.

Another major problem encountered in developing human monoclonal antibody producing hybridomas

Table 108.1. Human cell lines developed for human–human hybridoma production

Cell line	Cell type	Isotype production	Drug marker	Reference
LICR-LON-HMy2	LCL	IgG (κ)	8-AG	[32]
SKO-007	Myeloma	IgE (λ)	8-AG	[29]
TM-H2	Myeloma	IgG (κ)	6-TG	[36]
GM 1500 6TG-A11/A12	LCL	IgG (κ)	6-TG	[30]
KR-4	LCL	IgG (κ)	6-TG,Oua^R	[24]
H35.1.1.;0467.3	LCL	IgM (κ/λ)	8-AG	[35]
UC729-HF2	LCL	N.K.		[33]
KARPAS 707	Myeloma	λ		[28]

LCL—lymphoblastoid cell line.
6-TG—6-thioguanine.
8-AG—8-azaguanine.
Oua^R—ouabain resistant.
N.K.—not known.

is the difficulty in obtaining specifically immunized B lymphocytes. *In vitro* immunization protocols for sensitization of B cells isolated from peripheral blood have been developed [40–44]. Although these procedures have been successful for sensitizing B cells to cellular antigens, the antibodies generated are generally of low affinity due to the failure to obtain a secondary immune response after *in vitro* priming. *In vivo* immunization with subsequent isolation of peripheral blood lymphocytes or spleen cells for fusion is also successful, but limited to selected antigens for which immunization of humans is ethical. Several groups are currently attempting to exploit ongoing humoral immune responses in patients with autoimmune diseases or malignancies, or who are convalescing from viral and related illnesses, isolating peripheral blood lymphocytes from such individuals for hybridoma fusion to generate potentially interesting and useful human monoclonal-producing cell lines.

Screening hybrid colonies for specific antibody production

The frequency of clones producing monoclonal antibody of desired specificity may be quite low. Screening techniques to identify positive wells must be rapid and reproducible. These techniques will vary according to the desired antibody specificity and the intended use of the clonal product.

Detection of antibody to soluble antigen

A sensitive technique for detecting antibody to soluble antigen is the solid-phase radioimmunoassay. For this, approximately 0.1 ml of cells at $5–10 \times 10^6$ cells/ml are plated into each well of a 96-well microtitre plate at the completion of a hybridoma fusion. After individual hybrid cells have matured into colonies, the supernates from individual wells are tested for the presence of antigen-specific antibody. The authors generally test supernates diluted 1:5 in buffer containing 1% BSA as carrier-protein. This greatly reduces the number of false-positive wells that are identified during the initial screen. Testing is performed on antigen-coated polystyrene microtitre plates, as described in the chapter on solid-phase radioimmunoassay (Chapter 34). Cells from wells identified as having the desired monoclonal antibody are subsequently expanded and cloned using limiting dilution techniques or the FACS, as described below.

Detection of antibody to cell surface antigens

The radioimmunoassay technique mentioned above may be modified to screen for antibody to cell surface antigen. For this, the polystyrene microtitre plates are coated with solubilized membrane extract. Extract is prepared as described above and serially diluted with phosphate-buffered saline containing 1% BSA for coating the polystyrene plates. Coating is performed by adding 50 μl of extract to each well of the plates and allowing this to incubate at 4 °C overnight. Some investigators recommend treating the coated plates with 0.02% glutaraldehyde in PBS for 10 min at room temperature prior to washing away the unbound extract with 1% BSA in PBS [45]. The dilution of extract giving the lowest background with the highest signal needs to be empirically determined for each antigen tested.

The fluorescence activated cell sorter (FACS) and hybridoma technology

The FACS provides a highly useful tool for screening and cloning hybridomas producing desired monoclonal protein [46]. This section lists several applications of the FACS that have facilitated the authors' work with hybridomas.

Indirect cell surface staining

A useful method for detecting monoclonal antibody to surface antigens is to screen for indirect cell surface staining. Cells bearing the antigen of interest are incubated with hybridoma supernate with 0.1% NaN_3 at 4 °C for 20 min. The cells are then washed three times with staining medium, (phenol red-deficient RPMI-1640, 4% filtered fetal calf serum and 0.1% NaN_3). A second-step, fluorescein-conjugated, heterologous anti-immunoglobulin reagent, diluted to appropriate concentration in staining medium, is added to the cells for a second 20 min incubation. The sample is washed again three times in staining medium prior to analysis on the FACS. To facilitate testing a large number of hybridoma supernates, the authors routinely perform this assay using 96-well polystyrene microtitre plates. They distribute target cells into individual wells and perform subsequent staining and washing steps in these plates. The authors perform FACS analysis using a microsampling device that collects cells from individual wells of the polystyrene plates. This greatly facilitates screening of a large number of individual hybridoma supernates with minimal effort.

This approach has several advantages over more conventional screening procedures. It avoids the problems associated with extraction techniques for surface antigens described above, including disruption of antigenic determinates by detergent and partial digestion of membrane proteins through proteases released